Aust. J. Biol. Sci. **33**, 1980, 27–34

Immunoglobulin G Antibodies to the Antigens of *Lucilia cuprina* in the Sera of Fly-struck Sheep

I. J. O'Donnell,[A] *P. E. Green,*[B] *J. A. Connell*[B] *and P. S. Hopkins*[B]

[A]Division of Protein Chemistry, CSIRO, Parkville, Vic. 3052.
[B]Queensland Department of Primary Industries, Brisbane, Qld 4001.

Abstract

A solid-phase radioimmunoassay was used to demonstrate that sheep with myiasis caused by the larvae of the Australian sheep blowfly, *L. cuprina*, had serum IgG antibodies to antigens present in an extract of the ground-up larvae. Previously struck animals demonstrated a more severe myiasis than their unstruck counterparts when both groups were subjected to a standard larval challenge. The effects of immunosuppressive therapy were expressed in terms of a decrease in the total number of larvae growing to maturity and in the area of fly strike produced.

Introduction

Fly strike, i.e. invasion by myiasis-producing dipterous larvae, of sheep in Australia is a major problem of the sheep industry and, because of the development of resistance to insecticides (Shanahan and Roxburgh 1974), many lines of research are being followed in the hope of being able to control it. Among these are studies on factors which predispose to body strike, e.g. bacterial activity, fleece rot (Lipson 1978), oviposition stimulants (Merritt and Watts 1978); genetic manipulation of *Lucilia cuprina* to develop strains that will be blind or non-fertile in the field (Foster *et al.* 1978); and genetic improvement of Merino sheep to increase resistance to strike (McGuirk *et al.* 1978).

The work reported here is the beginning of an investigation into the biological expression of susceptibility to, and the possibility of immunizing sheep against, fly strike. We were unable to find in the literature any documented experimental attempts at immunization. In fact the only reference to such thoughts was by MacKerras (1936) in the following discouraging words—'It has also been suggested that sheep might be immunized against attack by the fly, in much the same way that they may be immunized against various bacterial diseases. This possibility has been examined but the experimental evidence so far is decidedly against it'. The present work reports the use of radioimmunoassays to demonstrate that immunoglobulin (IgG) antibodies to the antigens of the *L. cuprina* larvae do exist in the sera of struck sheep. It also examines the extent of myiasis in previously struck and non-struck sheep exposed to standard larval challenges and the effect of immunosuppression on the extent of this myiasis.

Experimental

Collection of Larvae of L. cuprina

For most experiments a mixture of second- and early third-stage larvae were used. They had been grown from Queensland field strains in fortified rabbit sera [0·5% (w/v) dipotassium hydrogen

orthophosphate and 2% (w/v) yeast extract]. Both OP (organophosphorous)-susceptible and OP-resistant strains were grown. Another batch was obtained from the Department of Genetics, University of Melbourne. These had been grown on beef livers. The larvae were either used fresh or stored at −70°C.

Extraction of Larval Antigens

The somatic larval antigens were extracted with a Potter–Elvejhem homogenizer. One gram of a mixture of the Queensland OP-resistant and OP-sensitive strains of larvae was extracted with 2·5 ml phosphate-buffered saline (PBS) at pH 7·2 containing 10^{-5}M phenylthiourea (to minimize monophenol monooxygenase (tyrosinase) activity), 10^{-3}M phenylmethylsulfonyl fluoride (to inhibit serine esterase activity) and 5×10^{-3}M ethylenediaminetetraacetic acid (disodium salt) to help minimize other unwanted enzyme activity. The extract was centrifuged, dialysed against PBS in 18/32 Visking cellulose tubing and stored frozen. The protein content, as judged by amino acid analysis of acid hydrolysates, before dialysis was 15–16 mg/ml and after dialysis 7–8 mg/ml.

The other extract studied was ICI-V (I.C.I. Melbourne, Aust.), a mixture of excretory–secretory (ES) antigens from *L. cuprina.*

Characterization of Antigens

The characterization of the antigens by cellulose acetate electrophoresis, isoelectric focusing, electrophoresis in SDS acrylamide gradient gels, and their fractionation on Sephadex G200 or DEAE-cellulose were carried out essentially as described by O'Donnell and Mitchell (1978). Disc gel electrophoresis was carried out using the conditions of Kinnear and Thompson (1975).

Sources of Sera

The sera used were:

(1) N.S.S.—normal sheep sera provided by the Division of Animal Health, CSIRO, Melbourne. They came from sheep which had never experienced attack by *L. cuprina.*
(2) C.P.1 and C.P.2—control pooled sera 1 and 2. These came from sheep from the field (in Queensland) which had no apparent history of fly strike.
(3) S.P.1 and S.P.2—struck pooled sera 1 and 2. These came from sheep which had been struck in the field (in Queensland) and had myiasis when the sera was taken from them.
(4) Recovered sheep—sera taken from a single sheep which had been struck (in Queensland) and had recovered spontaneously.
(5) The A-2, A-4, A-15, B-1, B-5, B-14, C-6, C-9, C-13, D-10, D-11, D-12 sera (Fig. 2) were from individual sheep which were being processed for an experiment on the effect of immunosuppressive agents on the severity of myiasis in sheep which had been struck (A and D) and which had not been struck (B and C) previously. Larval implants were undertaken on day 0 using the technique of Riches and O'Sullivan (1955). Samples of blood for radioimmunoassay of antibodies to *L. cuprina* antigens were taken 6 and 24 days later. The larval implant challenges were positive in all sheep.
(6) I.C.I.1 was serum from a sheep which had been repeatedly struck with *L. cuprina.*

Preparation of Rabbit Anti-sheep IgG

Rabbit antibody to sheep γ-globulin was bought from Calbiochem (U.S.A.) in 5-ml aliquots. The IgG was separated on a column of Protein A-Sepharose (Pharmacia, Sweden). The specific antibody was obtained by passage of this IgG through an affinity column of sheep immunoglobulin (prepared by precipitation with ammonium sulfate) bound to CNBr-Sepharose according to the manufacturer's instructions (Pharmacia, Sweden), and operated by the methods of Johnson and Garvey (1977). The material which came off in the glycine buffer at pH 2·8 contained all of the anti-sheep IgG as determined by immunoelectrophoresis.

Iodination of Rabbit Anti-sheep IgG and L. cuprina *Antigens with* ^{125}I

The methods employed for labelling the proteins with ^{125}I were those of Greenwood *et al.* (1963) using chloramine T. Carrier-free ^{125}I, at a concentration of 100 mCi/ml, was purchased from the Radiochemical Centre, Amersham, England.

Immunoprecipitation with Protein A-Sepharose

50 μl ^{125}I-labelled *L. cuprina* extract (4–5 ng; 50 000–60 000 cpm) were mixed with 50 μl serum from struck or unstruck sheep and 35 μl incubation buffer in 3·5-ml clear polystyrene tubes. After ½–1 h at 37°C, 200 μl (containing 10 mg) of a suspension of Protein A-Sepharose CL-4B (Pharmacia, Sweden) in incubation buffer was added (O'Donnell and Mitchell 1979). After 10 min standing the mixture was centrifuged and washed four times with PBS before counting in a Packard Auto-Gamma scintillation counter. Incubation buffer was 0·2% (w/v) bovine plasma albumin (Calbiochem, recrystallized A grade), 1% (v/v) Tween 20 and 0·1% (w/v) sodium azide in PBS.

Solid-phase Radioimmunoassay using Microtitre Trays

The *L. cuprina* antigens were stored frozen as a 0·8% (w/v) solution in PBS. This solution was only diluted (with PBS) immediately prior to its use to avoid adsorption losses on the sides of the tube (Elleman and Raison, unpublished data). A study of the effect of antigen concentration on the uptake of second antibody, i.e. rabbit anti-sheep IgG, showed that a concentration of 25 μg/ml was a value on the plateau. This value was that chosen for all experiments. *L. cuprina* antigen mixture in PBS was put in 25-μl aliquots into the wells of a polyvinyl chloride U-bottom microtitre tray (Cooke, Cat. No. 1-220-24, from Dyna-Tech, Virginia, U.S.A.) and dried under vacuum at 40°C for 2 h. Triplicates were done for each point. The antigen was fixed by the addition of 50 μl 10% (w/v) formalin in PBS for 15 min at 4°C and the plate washed twice with PBS. 50 μl of a protein 'filler' solution consisting of 1% (w/v) bovine plasma albumin (Calbiochem, recrystallized A grade), 1% (v/v) Tween 20, and 0·02% (w/v) sodium azide in PBS was added to each well and incubated for 30 min at 37°C. In later experiments de-activated normal rabbit serum, diluted 20 times, was used in place of the bovine plasma albumin. The filler solution was removed by inverting the plate and the wells were washed once with PBS. 25 μl of dilutions of serum from fly-struck and non-struck sheep in filler solution were then added to each well as triplicates and incubated for 2 h at 37°C. The serum samples were removed by aspiration and the plate washed three times with PBS. 25 μl (approx. 50 000 cpm) of second antibody, i.e. ^{125}I-labelled rabbit anti-sheep IgG diluted with filler solution to approximately 0·5 μg/ml, were added to each well and incubation carried out for 2 h at 37°C. The wells were emptied by aspiration and then washed four times with PBS. After drying at 37°C the individual wells were cut off and counted in a Packard Auto-Gamma scintillation counter. Control wells from which *L. cuprina* antigens or sheep sera were omitted were routinely included in all assays, as also was the positive serum, S.P.1 (from a pool of struck sheep).

In vivo *Studies*

The six struck (A and D) and six non-stuck sheep (B and C) used in the sera collection series were subdivided at random into treated and non-treated groups. Treatment consisted of the administration of immunosuppressive agents (Alkeran* 700 μg/kg subcutaneously on day −8 or Betsolan* 10 mg intramuscularly twice daily on days −1 to +3 inclusive). All animals were implanted with 1000 first-instar *L. cuprina* on day 0. Fully developed larvae were collected from each group and progressive larval counts monitored up until day 8. On day 8 the area of myiasis was shorn and measured.

Results

Quality and Stability of L. cuprina *Extracts*

Cellulose acetate and disc gel electrophoresis, gradient SDS gel electrophoresis and isoelectric focusing gave patterns of the *L. cuprina* extracts with discrete bands which did not change over a period of storage of the larval extracts of up to 2 months. Any pronounced enzyme attack on the extracted proteins by endogenous enzymes had apparently been satisfactorily prevented at the time of extraction.

*Alkeran and Betsolan are the Burroughs Wellcome, Australasia, and Glaxovet, Australia, preparations of melphalan and betamethasone, respectively.

Isoelectric focusing in the pH range 3·5–10 showed that most Coomassie-staining proteins of the larval extracts had low isoelectric points (pH 3–4).

Immunoprecipitation of ^{125}I-labelled L. cuprina*-IgG Complexes With Protein A-Sepharose*

In a limited study using this method it was found that sera from S.P.1 (struck sheep) gave 888 and 858 cpm (duplicates) and sera from I.C.I.1 (another struck sheep) had 644 and 606 cpm compared with values of 280–300 cpm for sera from control sheep. This method indicates the presence of antibodies to *L. cuprina* antigens in

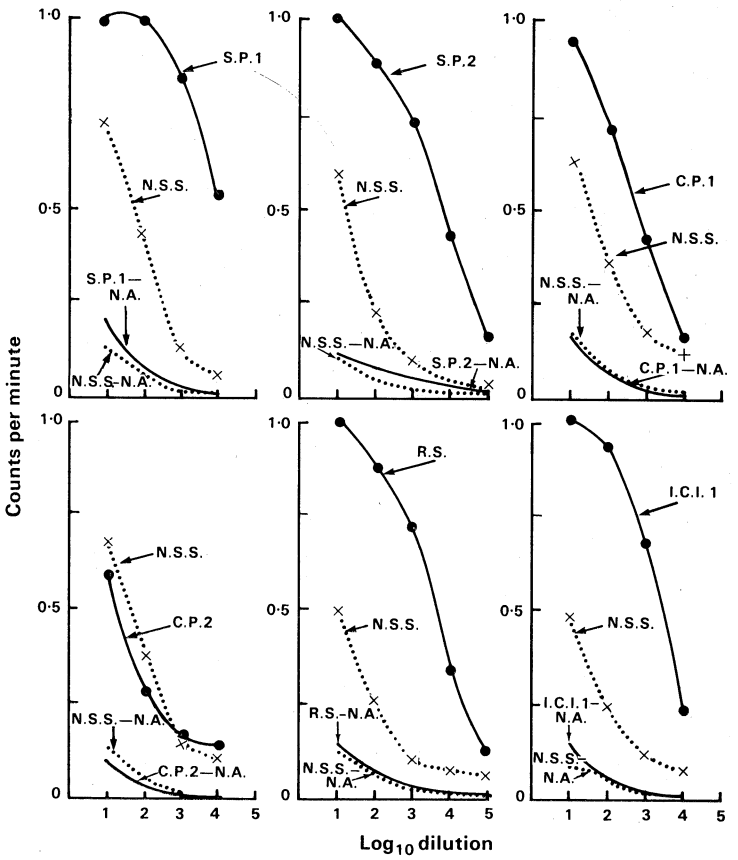

Fig. 1. Binding of ^{125}I-labelled rabbit anti-sheep IgG to specific sheep IgG (at varying dilutions of sheep sera—10^1, 10^2, 10^3, 10^4) against *L. cuprina* antigens bound to microtitre wells. ^{125}I counts were normalized to the maximum value obtained. This was for a particular sera, S.P.1, which at a dilution of 10, was used as a control in each experiment. S.P.1, S.P.2, I.C.I.1 and R.S. (recovered sheep) are from struck sheep or pools of struck sheep (see Experimental). C.P.1, C.P.2, and N.S.S. (normal sheep serum) are controls (see Experimental). N.A., no antigen in the wells of the microtitre plates. Individual experimental points were not put in on those graphs where they would be too crowded to distinguish from one another.

struck sheep even though the sensitivity of the method is probably greatly reduced by the fact that the major fraction of sheep IgG1 is not bound by Protein A-Sepharose (Goudswaard *et al.* 1978). The expense of Protein A-Sepharose CL-4B prevented its use for dilution curves as was done with assays using microtitre plates described next.

Solid-phase Radioimmunoassay using Microtitre Plates

The results of assays on a series of sera from struck and non-struck sheep are shown in Figs 1 and 2. It was found that the use of serial dilutions of the sera rather than a single dilution gave added confidence to the interpretation of the results. It is seen that all fly-struck sheep (S.P.1, S.P.2, recovered sheep, I.C.I.1—Fig. 1) and (A-2, A-4, A-15, D-10, D-11, D-12—Fig. 2) have antibodies to *L. cuprina* larval antigens. Boosting of the anti-larval antibodies by the larval implants was not detectable for sheep A-2, A-4, A-15 (Fig. 2*a*) and D-10, D-11, D-12 (Fig. 2*b*) in the period of observation used. The sheep whose sera comprised control pool 1 (C.P.1) were not struck at the time of collection of sera but obviously one (or some) of them still had antibodies in them from a previous strike.

In the vertical dimension (at serum dilution of 1 in 10^3) the number of counts per minute of fly-struck sheep were 2–8 times higher than the values for control sheep. In the horizontal dimension at the serum dilution values of sheep which give half the uptake of counts of the (standard) struck-sheep pooled sera, S.P.1, it can be seen that serum dilution values range from 10–100 for control sheep to 1000–10 000 for sera from struck sheep. Perhaps the differences could be made more marked if a somewhat 'cleaned-up' preparation of larval antigens were used.

Use of different larval extracts gave similar results. It did not matter whether excretory–secretory antigens, I.C.I. -V, or somatic antigens extracted from Melbourne or Brisbane strains of *L. cuprina* were used.

Preliminary experiments whereby the *L. cuprina* extracts were fractionated on a column of Sephadex G200 or DE52-cellulose and then assayed after binding each fraction to microtitre plates indicated that there were antigens right along the profiles, except that the salt peak off the G200 column contained very little or no antigens.

Table 1. Effects of immunosuppression and previous fly strike on the development of myiasis in sheep

Groups A and D had been struck previously (natural challenge); groups B and C had not been struck previously

Group	Treatment	Total larval count[A]	Average area of strike (cm^2)[B]
A	Alkeran	882 (29)	88 (66·5, 128, 70)
B	Betsolan	677 (23)	64 (73·5, 39·5, 79)
C	Nil	1009 (34)	125 (16·5, 169, 189)
D	Nil	1755 (59)	166 (199·5, 217·5, 80)

[A] Percentage received given in parentheses. These data are total values for each group of three animals.
[B] Actual values given in parentheses.

Table 1 presents the effects of immunosuppressive therapy on the development of myiasis. These data suggest that this type of treatment decreases the total number of larvae growing to maturity and the area of fly strike produced and will be investigated further. Apart from these effects the data, together with other non-systematic observations, also suggest that animals which had previously been struck subsequently demonstrated a more severe myiasis than their counterparts which were not previously struck.

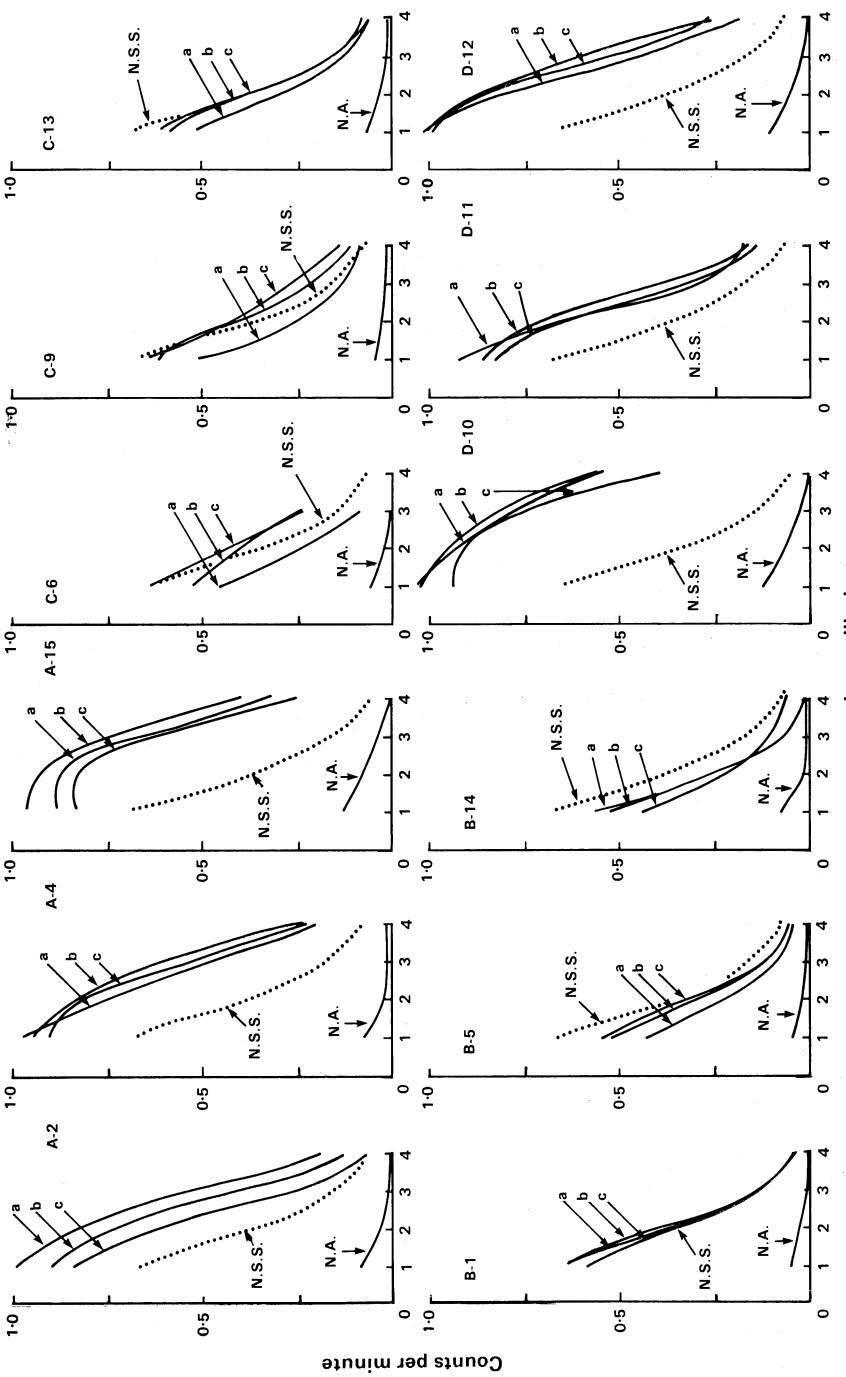

Fig. 2. Binding of ^{125}I-labelled rabbit anti-sheep IgG to specific sheep IgG (at varying dilutions of sheep sera—10^1, 10^2, 10^3, 10^4) against *L. cuprina* antigens bound to wells in microtitre plates. The ^{125}I counts were normalized to the maximum value obtained. This was for a particular serum, S.P.1, which at a dilution of 10 was used as a control in each experiment. The sheep in series A and D had previously been struck by *L. cuprina*. Those in series B and series C had not been previously struck. They were all implanted with *L. cuprina* larvae on day 0 (the *a* curves), and sera taken on day 6 (the *b* curves) and day 24 (the *c* curves). N.S.S., curve obtained for normal sheep serum. N.A., curves obtained with no antigen in the wells. Individual experimental points were not inserted on these graphs as they would be too crowded to distinguish from one another.

Discussion

The results reported here show that there are IgG antibodies to antigens present in *L. cuprina* larvae in fly-struck sheep. Their existence was demonstrable by radioimmunoassays but they were not present in large enough amounts to give precipitin lines in immunodiffusion experiments. The immunization of sheep against fly strike must therefore be considered a possibility and a goal worth pursuing further. This finding of IgG production against a surface invader is somewhat analagous to the production of antibodies to the causative agent, *Bacteroides nodosus*, of foot rot, another parasite whose invasion is a surface one (see Stewart 1978). The Old World screw-worm fly, a serious pest of the cattle industry in New Guinea, have larvae which bury much more deeply into the host than blowfly larvae, i.e. there is much more immunological contact. The work of D. P. A. Sands, Division of Entomology, CSIRO (see Annual Report of that Division for 1977–78, p. 54) has shown that these screw-worm larvae cause the production of precipitating antibodies in the host serum.

The antibodies being sought, i.e. IgG antibodies, did not appear quickly in sheep which had not been previously fly struck. Sheep B-1, B-5, B-14 and C-6, C-9, C-13 (Fig. 2) have no detectable IgG antibodies 24 days after larval implants. Any production of IgG antibodies must be later than this and so would be far too late to arrest any initial myiasis. We did not look for IgM antibodies which may be present in these early stages. The *in vivo* studies suggest that immunosuppression will reduce the severity of myiasis resulting from a standard larval challenge. The pooled data also show that sheep with a previous history of fly strike again express their susceptibility subsequent to a follow-up challenge. Both these findings would support the contention that the expression of sensitivity is at least partly associated with the mediation of an allergic response. Whether the IgE plasma profiles of susceptible and resistant sheep support this contention await elucidation.

The fact that there were antibodies present on day 0 in the sheep of series A and D did not prevent the taking of the larval implant. In the foot rot situation sheep normally have active infection and specific antibodies at the same time. However, if they are immunized with the bacteria (and presumably get an increase in antibodies specific for *B. nodosus*) the foot rot is cured (cf. Stewart 1978). By analogy the next experiment in this program of pursuit of a vaccine against *L. cuprina*-induced myiasis in sheep is to immunize sheep with *L. cuprina* antigens until a high level of specific antibody is induced, and then to challenge the sheep with *L. cuprina* larvae. Prior to the challenge experiment it will be necessary to study whether the sera from these immunized sheep will support normal larval growth when larvae are allowed to grow on agar plates containing sera.

Acknowledgments

We wish to thank Mr W. F. Williams and Mr C. C. Roberts for excellent technical assistance, Dr P. Outteridge and Dr N. Anderson, Division of Animal Health, CSIRO, for the gifts of normal sheep sera, Drs P. E. Montague, A. J. Campbell and S. Hogarth-Scott of I.C.I. Research Laboratories, Melbourne, for the gift of excretory–secretory larval antigens and sera from sheep which had been sequentially struck. Professor M. Whitten, Department of Genetics, University of Melbourne, provided the gift of live larvae. Part of the work was funded by the Wool Research Trust Fund.

References

Foster, G. G., Whitten, M. J., Vogt, W. G., Woodburn, T. L., and Arnold, J. T. (1978). Larval release method for genetic control of the Australian sheep blow-fly, *Lucilia cuprina* (Weideman), Diptera: Calliphoridae. *Bull. Entomol. Res.* **68**, 75–83.

Goudswaard, J., Van der Donk, J. A., Noordzij, A., Van Dam, R. H., and Vaerman, J. P. (1978). Protein A reactivity of various mammalian immunoglobulins. *Scand. J. Immunol.* **8**, 21–8.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific activity. *Biochem. J.* **89**, 114–23.

Johnson, G., and Garvey, J. S. (1977). Improved methods for separation and purification by affinity chromatography. *J. Immunol. Methods* **15**, 29–37.

Kinnear, J. F., and Thomson, J. A. (1975). Nature, origin and fate of major haemolymph proteins in *Calliphora*. *Insect. Biochem.* **5**, 531–52.

Lipson, M. (1978). The significance of certain fleece properties in susceptibility of sheep to fleece rot. *Wool Technol. Sheep Breed.* **16**, 27–32.

MacKerras, I. M. (1936). The sheep blowfly problem in Australia. CSIR Bull. No. 66. p. 37.

McGuirk, B. J., Atkins, K. D., Kowal, E., and Thornberry, K. (1978). Breeding for resistance to fleece rot and body strike—The Trangie programme. *Wool. Technol. Sheep Breed.* **26**, 17–24.

Merritt, G. C., and Watts, J. E. (1978). The changes in protein concentration and bacteria of fleece and skin during the development of fleece rot and body strike in sheep. *Aust. Vet. J.* **54**, 517–20.

O'Donnell, I. J., and Mitchell, G. F. (1978). An investigation of the allergens of *Ascaris lumbricoides* using a radioallergosorbent test (RAST) and sera of naturally infected humans: comparison with an allergen for mice identified by a passive cutaneous anaphylaxis test. *Aust. J. Biol. Sci.* **31**, 459–87.

O'Donnell, I. J., and Mitchell, G. F. (1980). An investigation of the allergens of *Ascaris lumbricoides* using a radioimmunoassay and sera of naturally infected humans. *Int. Archs. Allergy Appl. Immunol.* **61**, 213–19.

Riches, J. H., and O'Sullivan, P. J. (1955). The value of DDT, BHC, aldrin and dieldrin for the protection of sheep against body strike. *Aust. Vet. J.* **31**, 258–62.

Shanahan, G. J., and Roxburgh, N. A. (1974). The sequential development of insecticide resistance problems in *Lucilia cupriana* (Wied.) in Australia. *PANS* **20**, 190–202.

Stewart, D. J. (1978). The role of various antigenic factors of *Bacteriodes nodosus* in eliciting protection against foot rot in vaccinated sheep. *Res. Vet. Sci.* **24**, 14–19.

Manuscript received 14 September 1979, accepted 18 December 1979